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Quinolone Resistance Mediated by *norA*: Physiologic Characterization and Relationship to *flqB*, a Quinolone Resistance Locus on the *Staphylococcus aureus* Chromosome

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We identified a quinolone resistance locus, *flqB*, linked to transposon insertion Ω*1108* and *fus* on the *Sma*I D fragment of the *Staphylococcus aureus* NCTC 8325 chromosome, the same fragment that contains the *norA* gene. *S. aureus norA* cloned from *flqB* and *flqB*$^+$ strains in *Escherichia coli* differed only in a single nucleotide in the putative promoter region. There was no detectable change in the number of copies of *norA* on the chromosomes of *flqB* strains, but they had increased levels of *norA* transcripts. Cloned *norA* produced resistance to norfloxacin and other hydrophilic quinolones and reduced norfloxacin accumulation in intact cells that was energy dependent, suggesting active drug efflux as the mechanism of resistance. Drug efflux was studied by measurement of norfloxacin uptake into everted inner membrane vesicles prepared from *norA*-containing *E. coli* cells. Vesicles exhibited norfloxacin uptake after the addition of lactate or NADH, and this uptake was abolished by carbonyl cyanide *m*-chlorophenylhydrazone and nigericin but not valinomycin, indicating that it was linked to the pH gradient across the cell membrane. Norfloxacin uptake into vesicles was also saturable, with an apparent K_m of 6 μM, a concentration between those that inhibit the growth of *flqB* and *flqB*$^+$ *S. aureus* cells, indicating that drug uptake is mediated by a carrier with a high apparent affinity for norfloxacin. Ciprofloxacin and ofloxacin competitively inhibited norfloxacin uptake into vesicles. Reserpine, which inhibits the multidrug efflux mediated by the *bmr* gene of *Bacillus subtilis*, which is similar to *norA*, abolished norfloxacin uptake into vesicles as well as the norfloxacin resistance of an *flqB* mutant, suggesting a potential means for circumventing quinolone resistance as a result of drug efflux in *S. aureus*. These findings indicate that the chromosomal *flqB* resistance locus is associated with increased levels of expression of *norA* and strongly suggest that the NorA protein itself functions as a drug transporter that is coupled to the proton gradient across the cell membrane.

Fluoroquinolones such as norfloxacin, ciprofloxacin, and ofloxacin are broad-spectrum, synthetic antimicrobial agents that are widely used for the treatment of bacterial infections. Development of drug resistance in some species, particularly *Pseudomonas aeruginosa* and *Staphylococcus aureus*, has, however, limited the utilities of fluoroquinolones in some clinical settings. The genetics and mechanisms of bacterial fluoroquinolone resistance have been studied most extensively in *Escherichia coli* and *P. aeruginosa* (9). These drugs act on DNA gyrase, and in *E. coli*, mutations in the *gyrA* and *gyrB* genes encoding the DNA gyrase A and B subunits, respectively, have been shown to cause resistance (6, 11, 38, 40). In addition, mutations in genes that affect the expression of porin outer membrane proteins have been shown to cause resistance and to be associated with reduced levels of drug accumulation in intact cells (10, 11). Moreover, in these mutant gram-negative bacteria, reduced levels of drug accumulation appear to be dependent on energy, because drug accumulation returns to wild-type levels after treatment with protonophores such as dinitrophenol or carbonyl cyanide *m*-chlorophenylhydrazone (CCCP). One mechanism of reduced drug accumulation that is energy dependent is active drug efflux, as has been demonstrated for tetracycline (7, 37). In everted inner membrane vesicles prepared from both wild-type and mutant *E. coli*, saturable (K_m = 200 μM) uptake of norfloxacin was demonstrated after the addition of lactate or NADH (4). These findings suggested that the inner membrane of *E. coli* has an efflux transporter for norfloxacin. The gene(s) mediating this transport, however, has not yet been identified, leaving uncertain the role of drug efflux in the resistance phenotype.

Less is known about the genetics and mechanisms of quinolone resistance in *S. aureus*. At least three different loci appear to be involved in resistance. First, mutations in *S. aureus gyrA*, which are similar to those found in mutant *E. coli*, have been reported in resistant clinical isolates of *S. aureus* (33). Second, a resistance locus between *thrB* and *trp* on the *Sma*I A fragment of the *S. aureus* NCTC 8325 chromosome, which is distinct from *gyrA* and *gyrB*, has been found, but the mechanism of this resistance has not yet been defined (36). Third, the *norA* gene, which is located on the *Sma*I D fragment of the *S. aureus* chromosome (36), has been cloned in *E. coli* from quinolone-resistant but genetically undefined clinical strains of *S. aureus* (12, 26, 39). The amino acid sequence of NorA deduced from the nucleotide sequence predicts a hydrophobic protein with 12 membrane-spanning domains (39). Cloned *norA* in *E. coli* and *S. aureus* produces resistance to hydrophilic more than hydrophobic quinolones and reduced levels of drug accumulation in intact *E. coli* cells (12, 39). This reduced level of accumulation is also reversed by CCCP, suggesting that NorA is involved in quinolone efflux transport.

We report here a new chromosomal quinolone resistance locus, *flqB*, which is linked to *fus* and the transposon insertion

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TABLE 1. Bacterial strains used in the study

Strain	Genotype	Origin or reference
S. aureus		
ISP794	8325[a] *pig-131*	34
ISP1461	8325 *pig-131 nov-142 fus-149 pur-140* Ω(*chr*::Tn*551*)*1030* Ω(*chr*::Tn*916*)*1108*	28
MT1222	8325 *pig-131 flqA flqB flqC*	36
MT5222	8325 *pig-131 nov-142 hisG15 flqA541*	36
MT5224c9	8325 *pig-131 nov-142 hisG15 flqA543*	36
MT21713	8325 *pig-131 flqA flqB flqC fus-149* Ω(*chr*::Tn*916*)*1108*	This study; ISP1461 DNA × MT1222
MT23142	8325 *pig-131 flqB* Ω(*chr*::Tn*916*)*1108*	This study; MT21713 DNA × ISP794
E. coli DH10B	F⁻ *araD139* Δ(*ara leu*)*7697* Δ*lacX74 galU galK mcrA* (*mrr-hsdRMS-mcrBC*) *rpsL dor* Φ80d*lac*ZM15 *endA1 nupG recA1*	Bethesda Research Laboratories

[a] *S. aureus* phage group III strain NCTC 8325 (27).

Ω*1108* on the *Sma*I D fragment, and the relationship of this locus to the increased levels of expression of the *norA* gene. We further report the characteristics of *norA*-mediated transport in everted inner membrane vesicles, indicating that *norA* effects saturable efflux transport of norfloxacin, which is coupled to the pH gradient across the vesicle membrane. Norfloxacin transport is also shown to be competitively inhibited by other quinolones and is also inhibited by reserpine, which reverses the resistance phenotype of the *flqB* mutant.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in the study are listed in Table 1.

Media and chemicals. Brain heart infusion and Penassay broth were from Difco Laboratories (Detroit, Mich.), and Mueller-Hinton II (MH) agar and Trypticase soy broth and agar were from Becton-Dickinson Microbiology Systems (Cockeysville, Md.). Luria-Bertani (LB) agar was as described previously (19).

Lysostaphin was purchased from ICN Biomedicals, Inc. (Costa Mesa, Calif.). Norfloxacin, ciprofloxacin, ampicillin, carbenicillin, tetracycline, erythromycin, fusidic acid, reserpine, verapamil, and CCCP were purchased from Sigma Chemical Co. (St. Louis, Mo.). Ofloxacin was obtained from Ortho Pharmaceutical Corporation (Raritan, N.J.), and sparfloxacin was obtained from Parke-Davis Pharmaceutical Research Division (Ann Arbor, Mich.). [^{3}H]norfloxacin (uniformly labeled in the piperazine ring; 15.1 Ci/mmol) was generously provided by Merck Sharp & Dohme Research Laboratories (Rahway, N.J.).

Drug susceptibility determinations. MICs were determined by agar dilution on either MH agar or Trypticase soy agar containing serial twofold dilutions of drug. The effect of reserpine on quinolone susceptibility was determined by broth dilution in Penassay broth as described previously (21). Incubations were done at 35 or 37°C, and growth was scored at 24 and 48 h.

Transformations. For transformation in *S. aureus*, high-molecular-weight genomic DNA was prepared by the method of Stahl and Pattee (34), and cells were made competent for transformation as described previously (16). Selections for Tn*916* in *S. aureus* were performed with 10 μg of tetracycline per ml.

Transformations in *E. coli* DH10B were performed by standard procedures (30), with selections for resistance performed on agar containing 100 μg of ampicillin per ml and 0.125 μg of norfloxacin per ml.

Amplification of *norA* by PCR. An oligonucleotide primer pair (oligomers NorAL1 5′-CCG GAA TTC CGG GCT CGT CAA TTC CAG TGG CTC ATG-3′ and NorAR1 5′-CCG GAA TTC CGG TGT CAT CCC CTT ACC CAC ATT TCC-3′) was synthesized on the basis of sequences 0.5 kb upstream and 0.3 kb downstream from the *norA*-coding sequence (39), with *Eco*RI sites (underlined) appended at the 5′ ends. Vent DNA polymerase (New England Biolabs) was used with these oligonucleotides to amplify *norA* DNA from *S. aureus* ISP794 and MT1222 by PCRs for 30 cycles. Reaction mixtures contained 6 mM $MgSO_4$ and were prepared according to the manufacturer's guidelines for primer extension. For each cycle, the DNA was denatured at 94°C for 45 s, reannealed at 68°C for 45 s, and extended at 75°C for 2 min each.

Cloning of *norA*. A 5.3-kb *Hin*dIII fragment containing *norA* was cloned in pUC19 in *E. coli* DH10B from genomic DNA prepared from *S. aureus* ISP794 and MT1222 by the method of Matsuhashi et al. (17). Clones were restriction mapped to determine the orientation of the inserted genomic DNA, and two plasmid clones, pMT101 (from MT1222) and pMT102 (from ISP794), which were oriented similarly with respect to the plasmid, were studied further. The 1.9-kb *norA* PCR products were cloned into the *Eco*RI site of pGEM7zf(+), generating plasmid pEN201 from strain MT1222 and plasmid pEN202 from strain ISP794. Clones were selected on LB agar containing carbenicillin (100 μg/ml) and norfloxacin (0.125 μg/ml).

Nucleotide sequencing of *norA*. Nucleotide sequences were determined directly from double-stranded plasmid DNA by the chain termination method (31) by using Sequenase 2.0 (U.S. Biochemicals) and a series of nested oligonucleotide primers for both the coding and complementary strands. The sequences of both DNA strands of pMT101 and pMT102 and the coding strands of pEN201 and pEN202 were determined.

Southern hybridizations. Genomic DNAs from MT1222, MT23142, and ISP794 were digested with either *Eco*RI or *Hin*dIII, electrophoresed, and transferred to GeneScreen Plus (DuPont) membranes by the method of Southern (32). The membranes were probed with a 4.3-kb *Kpn*I-*Hin*dIII *norA*-containing fragment from MT23142 that was labeled with [α-^{32}P]dCTP by using the Random Prime Kit (Boehringer Mannheim). Hybridization and washes were done according to the recommendations of the manufacturer of GeneScreen Plus by using the formamide procedure at 42°C.

TABLE 2. Linkage of *flqB* with *fus* and *Ω1108* determined by transformation

Donor		Recipient		Transformants selected for *Ω1108*	
Strain	Genotype	Strain	Genotype	Class	No. in class/ total no. (%)
ISP1461	*Ω1108 fus*	MT1222	*flqB flqA flqC*	*flqB*$^+$ *fus*$^+$	17/397 (4.2)
				flqB fus	1/397 (0.2)
MT21713	*Ω1108 fus flqB*	ISP794	Wild type	*flqB*$^+$ *fus*$^+$	215/258 (83)
				flqB fus$^+$	22/258 (8.5)
				flqB$^+$ *fus*	16/258 (6.2)
				flqB fus	5/258 (1.9)

Northern hybridizations. Total cellular RNA was prepared from cells of *E. coli* and *S. aureus* ISP794, MT1222, and MT23142 by the hot phenol method (1). To effect cell lysis, *S. aureus* cells were incubated with lysostaphin (400 μg/ml) for 10 min at 35°C immediately prior to initiating the hybridization procedure. A total of 20 μg of RNA from each strain was separated on an agarose-formaldehyde gel (14) and was transferred onto a GeneScreen Plus membrane for Northern analysis. The *norA* DNA probe was prepared by PCR and was labeled with ^{32}P by the random prime method as indicated above. Hybridization and washes were done as described above.

Preparation of everted inner membrane vesicles. Everted inner membrane vesicles were prepared from *E. coli* DH10B containing various plasmid constructs as described previously (4), except that the vesicles were washed a second time with 50 mM KPO_4 (pH 6.6) before collection and resuspension of the final pellet. In experiments to determine the requirement for magnesium, vesicles were washed twice in 50 mM KPO_4 (pH 6.6)–10 mM EDTA.

Measurement of norfloxacin accumulation by intact *E. coli* and everted inner membrane vesicles. Measurement of the accumulation of [^{3}H]norfloxacin by intact *E. coli* cells was similar to that described previously (10). For standard assays of norfloxacin uptake by everted membrane vesicles, incubations were performed at 30°C, and vesicles were diluted to 0.5 mg of protein per ml into assay buffer (50 mM KPO_4 [pH 7.5], 1 mM $MgCl_2$). [^{3}H]norfloxacin (2.0 μM, 0.74 Ci/mmol) was added at time zero, lithium lactate (20 mM) or NADH (5 mM) was added at 3 min, and CCCP (100 μM) was added at 10 min. Samples (25 μl) were rapidly diluted into 5 ml of 0.1 M KPO_4 (pH 7.5)–0.1 M LiCl, collected under vacuum on Metricel filters (Gelman), and washed with 4 ml of 0.1 M KPO_4 (pH 7.5)–0.1 M LiCl. Filters were dried and counted by liquid scintillation in toluene-Omnifluor (DuPont). Energy-dependent uptake was determined by subtraction of the counts of controls from which lactate and NADH were omitted or to which CCCP was added. In some experiments, nigericin, valinomycin, reserpine, or verapamil was added 5 min prior to the addition of [^{3}H]norfloxacin. All experiments were performed at least twice.

The kinetics of norfloxacin uptake into everted vesicles were determined at 20°C. Vesicles were incubated for 5 min in buffer containing lactate. [^{3}H]norfloxacin was added at time zero, and samples were collected as described above within 10 s and then approximately every 30 s thereafter for up to 3 min. CCCP was added at 4 min, and additional samples were taken at 5 and 7 min. The final total concentrations of norfloxacin ranged from 0.5 to 25 μM, with a constant amount of [^{3}H]norfloxacin (1.4 μCi/ml, 94 nM). At each norfloxacin concentration, uptake increased with time in the presence of lactate. In contrast, in the absence of lactate, norfloxacin uptake was substantially lower and was stable between 1 and 3 min. The average of the uptake values in the absence of lactate was subtracted from the uptake values in the presence of lactate to estimate lactate-dependent uptake. V_{max} and apparent K_m values were estimated from the plots of the initial rate of lactate-dependent uptake (V) versus norfloxacin concentration (S) and from plots of $1/V$ versus $1/S$ and V versus V/S. The counting efficiency for [^{3}H]norfloxacin was determined in triplicate by counting filters that contained unlabeled vesicles and that were spotted with a known amount of [^{3}H]norfloxacin.

For competition experiments with other quinolones, a fixed amount of unlabeled ciprofloxacin (5 or 15 μM) or ofloxacin (15 or 45 μM) was added to each member of the series of labeled norfloxacin stock solutions of different specific activities. Competitive inhibition was determined by the concurrence of the y intercepts ($1/V_{max}$) from plots of $1/V$ versus $1/S$. Apparent K_i values were calculated by comparing K_m values in the presence and absence of the inhibitor by the equation $K_i = [I]/(K_m^i/K_m^0 - 1)$, in which K_m^i and K_m^0 are the apparent K_m values in the presence and absence, respectively, of an inhibitor of concentration I.

Measurement of the effect of norfloxacin on lactate-induced changes in acridine fluorescence in everted vesicles. Everted vesicles were prepared in uptake assay buffer containing acridine orange (2 μM) in the presence and absence of norfloxacin (400 μM) as described previously (4). Quenching of acridine fluorescence upon the addition of lactate was monitored at excitation and emission wavelengths of 490 and 530 nm, respectively, with a SPEX Fluorolog 2 series spectrofluorimeter by using SPEX dm3000 software (version 2.5).

RESULTS

Identification and mapping of the *flqB* locus. *S. aureus* MT1222 is a highly resistant mutant of strain ISP794 selected by serial passage on increasing concentrations of norfloxacin (36). Strain ISP1461 contains two markers on the chromosomal *Sma*I D fragment, *fus*, which encodes resistance to fusidic acid, and *Ω1108*(Tn*916*), a transposon insertion encoding resistance to tetracycline (27) (Table 1). High-molecular-weight genomic DNA from ISP1461 was used to transform MT1222, selecting for Tn*916* (Table 2). For 18 of 397 transformants (4.5%) there was an eightfold reduction in the MIC of ciprofloxacin, from 64 μg/ml (that for MT1222) to 8 μg/ml, indicating the presence of a locus linked to *Ω1108* that contributes to fluoroquinolone resistance. We named this locus *flqB*. DNA was then prepared from a resistant transformant MT21713 [*flqB* (and other resistance loci) *fus Ω1108*(Tn*916*)] (MIC, 64 μg of ciprofloxacin per ml), and this DNA was used to transform wild-type strain ISP794 (MIC, 0.25 μg of ciprofloxacin per ml), selecting for Tn*916*. For 27 of

TABLE 3. Quinolone susceptibilities of *S. aureus* and *E. coli* strains containing cloned *norA*

Strain	Genotype	MIC (μg/ml)			
		Norfloxacin	Ciprofloxacin	Ofloxacin	Sparfloxacin
S. aureus					
ISP794	Wild type	0.5	0.25	0.5	0.125
MT1222	*flqB flqA flqC*	256.0	64.0	8.0	8.0
MT23142	*flqB Ω1108*	4.0	1.0	0.5	0.125
E. coli DH10B with plasmid:					
pUC19 or pGEM7zf(+)		0.02	≤0.005	0.01	0.001
pMT101		0.64	0.04	0.04	0.001
pMT102		1.28	0.08	0.04	0.0025
pEN201		0.64	0.08	0.04	0.001
pEN202		0.64	0.08	0.04	0.001

258 transformants (10.5%) there was a fourfold increase in the ciprofloxacin MIC (1.0 μg/ml), and 21 of 258 transformants (8.1%) were resistant to fusidic acid. Because only five transformants (1.9%) had both *fus* and *flqB* markers, the findings suggest a gene order of *flqB Ω1108 fus*. These experiments established the location of *flqB* on the chromosomal *Sma*I D fragment and the ability of *flqB* to confer fluoroquinolone resistance. The original mutant strain MT1222 and one transformant, MT23142 (*flqB*, *Ω1108*), were used in subsequent experiments. MT1222 exhibited greater increments in resistance to norfloxacin and ciprofloxacin than to ofloxacin and sparfloxacin, and MT23142 exhibited increases in resistance only to norfloxacin and ciprofloxacin (Table 3).

Cloning of *norA* genes from wild-type and *flqB* strains. We showed that the *norA* gene, like *flqB*, is located on the *Sma*I D fragment. Previously, we reported cloning a fluoroquinolone resistance fragment from MT1222 *Hin*dIII-digested genomic DNA (36). The 5.3-kb fragment that we obtained had a restriction enzyme map consistent with those of the *norA* genes cloned by other laboratories (13, 40). The same cloning experiment (36) was performed with genomic DNA from wild-type strain ISP794. Unexpectedly, norfloxacin-resistant, carbenicillin-resistant clones were obtained at a frequency similar to that obtained with genomic DNA from MT1222. Additionally, all clones from both MT1222 and ISP794 contained a 5.3-kb insert. Two clones, pMT101 (from MT1222) and pMT102 (from ISP794), which contained inserts in the same orientations, were studied further (Fig. 1). The restriction map of the 5.3-kb fragment from ISP794 was again consistent with that of the *norA* gene. pMT101 and pMT102 conferred similar levels of resistance to fluoroquinolones (Table 3), with greater increments in resistance seen for norfloxacin and ciprofloxacin than for ofloxacin and sparfloxacin, findings which are consistent with those reported previously (39).

To confirm that it was the *norA* gene that was responsible for the resistance phenotype, we amplified *norA* from MT1222 and ISP794 by PCR using Vent polymerase. The PCR products were then ligated into the *Eco*RI site of vector pGEM7zf(+), and the vector was used to transform *E. coli* DH10B. Several clones and, in particular, pEN201 (from MT1222) and pEN202 (from ISP794) were studied further. Both conferred resistance to norfloxacin (Table 3), indicating that the cloned *norA* gene was sufficient to confer resistance at levels similar to those conferred by pMT101 and pMT102.

Comparison of the nucleotide sequences of *norA* from wild-type and *flqB* strains. To identify possible differences in the *norA* sequences between *flqB* and *flqB*$^+$ strains, we determined the complete nucleotide sequence of *norA*, including sequences 359 bp upstream and 247 bp downstream from the

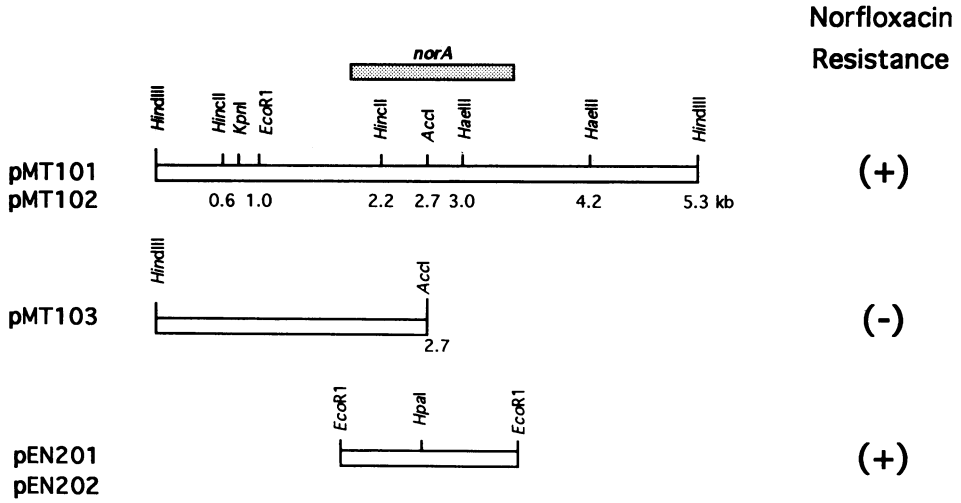

FIG. 1. Restriction enzyme sites of *S. aureus* DNA containing *norA* cloned from strain MT1222(pMT101, pEN201) and strain ISP794(pMT102, pEN202). pMT103 was constructed by subcloning the 2.7-kbp *Hin*dIII-*Acc*I fragment of pMT101 in pUC19.

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-359    ATTTTAATAC AACGTCATCA CATGCACCAA TGCCGCTGAC AGATGTAAAT
        GTTAAGTCTT GGTCATCTGC AAAGGTTGTT ATACATTCAA CGATATCTTC
        TCCTTTTTCC AACACTAGTA GTATAGTATG ATTACTTTTT TGCAATTTCA
        TATGATCAAT CCCCTTTATT TTAATATGTC ATTAATTATA CAATTAAATG
        GAAAATAGTG ATAATTACAA AGAAAAAATA TTGTCAAATG TAGCAATGTT
                                        [-35]
-109    GTAATACAAT ATAGAAACTT TTTACGAATA TTTAGCATGA ATTGCAATCT
          [-10]            G
-59     GTCGTGGAAA AGAAGAATAA CAGCTTTAAG CATGACATGG AGAAAAAAGA
                                                    [SD]

-9      GGTGAGCATA TGAATAAACA GATTTTTGTC TTATATTTTA ATATTTTCTT
                 M  N  K  Q   I  F  V    L  Y  F   N  I  F  L
42      GATTTTTTTA GGTATCGGTT TAGTAATACC AGTCTTGCCT GTTTATTTAA
          I  F  L   G  I  G   L  V  I  P   V  L  P   V  Y  L
92      AAGATTTGGG ATTAACTGGT AGTGATTTAG GATTACTAGT TGCTGCTTTT
        K  D  L  G   L  T  G   S  D  L   G  L  L  V   A  A  F
142     GCGTTATCTC AAATGATTAT ATCGCCGTTT GGTGGTACGC TAGCTGACAA
          A  L  S   Q  M  I  I   S  P  F   G  G  T   L  A  D  K
192     ATTAGGGAAG AAATTAATTA TATGTATAGG ATTAATTTTG TTTTCAGTGT
          L  G  K   K  L  I   I  C  I  G   L  I  L   F  S  V
242     CAGAATTTAT GTTTGCAGTT GGCCACAATT TTTCGGTATT GATGTTATCG
        S  E  F  M   F  A  V   G  H  N   F  S  V  L   M  L  S
292     AGAGTGATTG GTGGTATGAG TGCTGGTATG GTAATGCCTG GTGTGACAGG
          R  V  I   G  G  M  S   A  G  M   V  M  P   G  V  T  G
342     TTTAATAGCT GACATTTCAC CAAGCCATCA AAAAGCAAAA AACTTTGGCT
          L  I  A   D  I  S   P  S  H  Q   K  A  K   N  F  G
392     ACATGTCAGC GATTATCAAT TCTGGATTCA TTTTAGGACC AGGGATTGGT
          Y  M  S  A   I  I  N   S  G  F   I  L  G  P   G  I  G
442     GGATTTATGG CAGAAGTTTC ACATCGTATG CCATTTTACT TTGCAGGAGC
          G  F  M   A  E  V  S   H  R  M   P  F  Y   F  A  G  A
492     ATTAGGTATT CTAGCATTTA TAATGTCAAT TGTATTGATT CACGATCCGA
          L  G  I   L  A  F   I  M  S  I   V  L  I   H  D  P
542     AAAAGTCTAC GACAAGTGGT TTCCAAAAGT TAGAGCCACA ATTGCTAACG
        K  K  S  T   T  S  G   F  Q  K   L  E  P  Q   L  L  T
592     AAAATTAACT GGAAAGTGTT TATTACACCA GTTATTTTAA CACTTGTATT
          K  I  N   W  K  V  F   I  T  P   V  I  L   T  L  V  L
642     ATCGTTTGGT TTATCTGCAT TTGAAACATT GTATTCACTA TACACAGCTG
          S  F  G   L  S  A   F  E  T  L   Y  S  L   Y  T  A
692     ACAAGGTAAA TTATTCACCT AAAGATATTT CGATTGCTAT TACGGGTGGC
        D  K  V  N   Y  S  P   K  D  I   S  I  A  I   T  G  G
742     GGTATATTTG GGGCACTTTT CCAAATCTAT TTCTTCGATA AATTTATGAA
          G  I  F   G  A  L  F   Q  I  Y   F  F  D   K  F  M  K
792     GTATTTCTCA GAGTTAACAT TTATAGCTTG GTCATTATTA TATTCAGTTG
          Y  F  S   E  L  T   F  I  A  W   S  L  L   Y  S  V
842     TTGTCTTAAT ATTATTAGTT TTTGCTAATG ACTATTGGTC AATAATGTTA
        V  V  L  I   L  L  V   F  A  N   D  Y  W  S   I  M  L
892     ATCAGTTTTG TTGTCTTCAT AGGTTTTGAT ATGATACGAC CAGCCATTAC
          I  S  F   V  V  F  I   G  F  D   M  I  R   P  A  I  T
942     AAATTATTTT TCTAATATTG CTGGAGAAAG GCAAGGCTTT GCAGGCGGAT
          N  Y  F   S  N  I   A  G  E  R   Q  G  F   A  G  G
992     TGAACTCGAC ATTCACTAGT ATGGGTAATT TCATAGGTCC TTTAATCGCA
        L  N  S  T   F  T  S   M  G  N   F  I  G  P   L  I  A
1042    GGTGCGTTAT TTGATGTACA CATTGAAGCA CCAATTTATA TGGCTATAGG
          G  A  L   F  D  V  H   I  E  A   P  I  Y   M  A  I  G
1092    TGTTTCATTA GCAGGTGTTG TTATTGTTTT AATTGAAAAG CAACATAGAG
          V  S  L   A  G  V   V  I  V  L   I  E  K   Q  H  R
1142    CAAAATTGAA AGAACAAAAT ATGTAGCATA AGTATTTTGG TGTATATTGA
        A  K  L  K   E  Q  N   M  ***
1192    TATAAAGTAA AGCGTAATAT TATGAATGAT TAGCATCGTT TTTCTTATGA
        ATTTTATTAA GAAAATTCGA TGCTTTACAT TTAAAAAGAT TCGATTGACT
1292    AAATGTTTTA CTCTTTATAT TTAAATGTTA TATGTAACAA AAAATGATTT
        TGAGTAATAA ACATGTTACA AATATTACAT TCTTTTTAAA TTGCAATCCA
1392    CATACCTAAT TCATTAACGT TAATGTGTTA AGATGATAAA AAATGAGTAA
1442    GGAAATGTGG GTAAGGGGAT
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FIG. 2. Nucleotide sequence of 1,820 bp of *S. aureus* DNA containing the *norA* genes from ISP794 (complete sequence shown) and MT1222 (changes are indicated above the ISP794 sequence). The deduced amino acid sequence of NorA is given below the nucleotide sequence. The −35 and −10 sequences of the putative promoter, the Shine-Delgarno sequence, and a putative transcription terminator are underlined (39).

open reading frame in pMT101 and pMT102 (both strands) and pEN201 and pEN202 (coding strand) (Fig. 2). The sequences of pMT101 and pEN201 were identical, as were the sequences of pMT102 and pEN202. Sequences within the *norA* structural genes from MT1222 and ISP794 did not differ. A single nucleotide difference was identified 89 bp upstream from the putative ATG start codon: a G in mutant MT1222 replaced the T in wild-type ISP794.

***norA* mRNA levels in wild-type and *flqB* strains.** To compare *norA* expression in wild-type and *flqB* cells, equal amounts of total cellular RNA from *S. aureus* ISP794, MT1222, and MT23142 cells and *E. coli* DH10B cells containing plasmids pUC19, pMT101, and pMT102 were subjected to Northern analysis (Fig. 3). *norA* transcripts appeared in all of the *S.*

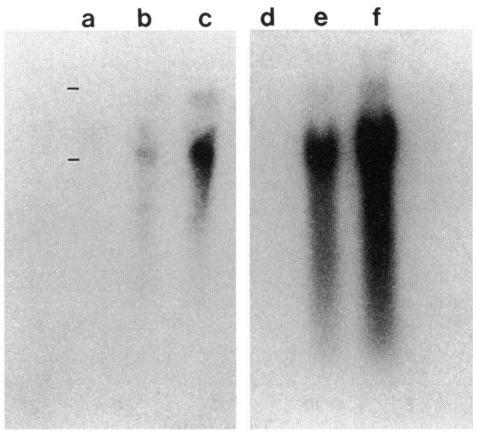

FIG. 3. Northern hybridization of total cellular RNA from *E. coli* DH10B containing pUC19 (lane d), pMT101 (lane e), and pMT102 (lane f) and from *S. aureus* ISP794 (lane a), MT1222 (lane b), and MT23142 (lane c) by using a *norA* DNA probe. The migrations of RNA size markers of 2.37 and 1.35 kb are indicated by the upper and lower hash marks, respectively. In the composite shown, the photographic exposure for lanes a to c was longer than that for lanes d to f.

aureus cells and the *norA*-containing *E. coli* cells, but no signal was seen in the *E. coli* DH10B(pUC19) control. In both *S. aureus* and *norA*-containing *E. coli*, primary and secondary messages of approximately 1.4 and 2.3 kb, respectively, were seen. An additional 2.9-kb message appeared in the *norA*-containing *E. coli* cells.

In *S. aureus*, *flqB* mutants showed substantially greater steady-state levels of *norA* message than wild-type ISP794; MT23142 (*flqB*) showed the highest levels; this was followed by MT1222 (*flqA*, *flqB*, *flqC*). *E. coli* containing cloned *norA* showed at least 10-fold higher levels of *norA* expression than any of the *S. aureus* strains. The differences in the *norA* transcripts in MT23142 and MT1222 suggest the possibility that the mutations (*flqA* and *flqC*) in MT1222, in addition to *flqB*, may also affect *norA* expression.

Effects of cloned *norA* on norfloxacin accumulation in intact cells. pMT101, which contains *norA*, but not pUC19 (Fig. 1), caused a reduced level of accumulation of norfloxacin by *E. coli* DH10B (Fig. 4). This reduced level of accumulation was reversed by the addition of the protonophore CCCP, suggesting that it is dependent on the presence of a proton motive force across the cell membrane. Such an energy-dependent and reduced level of norfloxacin accumulation produced by cloned *norA* suggests that *norA* mediates the active efflux of norfloxacin (15, 39), but this efflux has been studied in only a limited fashion in everted vesicle systems (13). To characterize *norA*-mediated norfloxacin efflux further, we characterized norfloxacin transport in everted inner membrane vesicles, in which norfloxacin efflux across the inner membrane can be measured directly as vesicle uptake.

Energetics of *norA*-mediated transport of norfloxacin into everted inner membrane vesicles. Accumulation of norfloxacin in everted vesicles from cells containing pMT101 was increased fivefold by the addition of lactate (Fig. 5). Similar levels of uptake were seen when NADH replaced lactate (data not shown). Under these conditions, lactate-dependent accumulation required the presence of *norA* and was not seen in the pUC19 control. This accumulation was also rapidly reversed by the addition of CCCP (100 μM), suggesting that uptake is coupled to the proton motive force generated across the

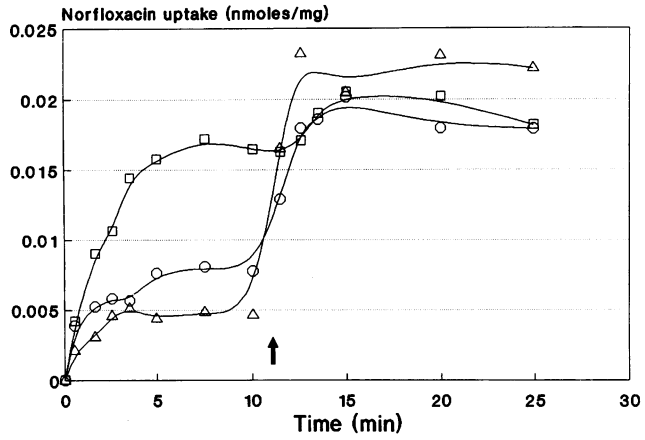

FIG. 4. Accumulation of norfloxacin in intact *E. coli* DH10B cells containing plasmids pUC19 (□), pMT101 (○), and pMT102 (△). Norfloxacin was added at time zero, and CCCP (100 μM) was added at the time indicated by the arrow. The increase in uptake followed the addition of CCCP.

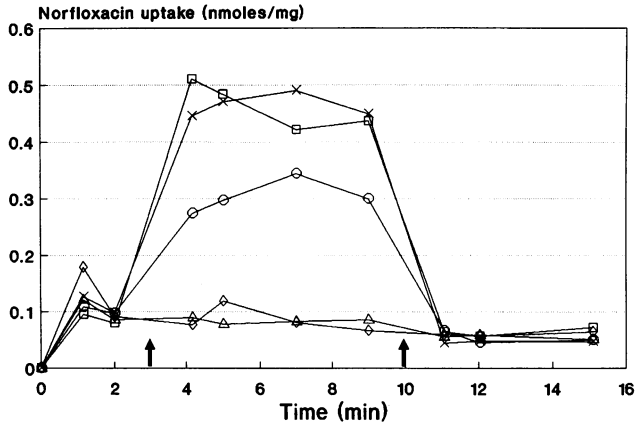

FIG. 6. Effects of inhibitors on uptake of norfloxacin by everted vesicles from *E. coli* DH10B(pMT101). The inhibitor nigericin (0.55 μM [◇] and 2.8 μM [△]) or valinomycin (0.36 μM [×] and 1.8 μM [□]) or the diluent control (○) was added at 5 min prior to the addition of norfloxacin at time zero. Lactate (20 mM) was added at the time indicated by the arrow on the left, and CCCP (100 μM) was added at the time indicated by the arrow on the right.

membrane after the addition of lactate. Lower concentrations of CCCP produced graded inhibition (10 μM, <1% of that of the no-drug control; 1.0 μM, 5.8 to 9.1% of that of the control; 0.1 μM, 38 to 84% of that of the control). To determine which of the components of the proton motive force, the proton (ΔpH) or the electrical (ΔΦ) gradient, drives uptake, we tested the effects of nigericin, which selectively collapses ΔpH, and valinomycin, which selectively collapses ΔΦ, using concentrations similar to those used previously in vesicle systems (4, 8). Because CCCP has been reported to have effects on norfloxacin accumulation in nonenergized artificial liposomes (5), these tests are also important for ruling out the artifactual effects of CCCP. Nigericin (0.55 and 2.8 μM) completely abolished norfloxacin uptake; lesser effects were seen at lower concentrations of nigericin (0.1 μM, 7% of that of the no-drug control; 0.01 μM, 88% of that of the no-drug control). In contrast, valinomycin (0.36 and 1.8 μM in the presence of excess K^+) produced a reproducible, slight increase in the level of accumulation (Fig. 6). Thus, *norA*-mediated norfloxacin uptake in everted vesicles appears to be specifically coupled to ΔpH across the membrane.

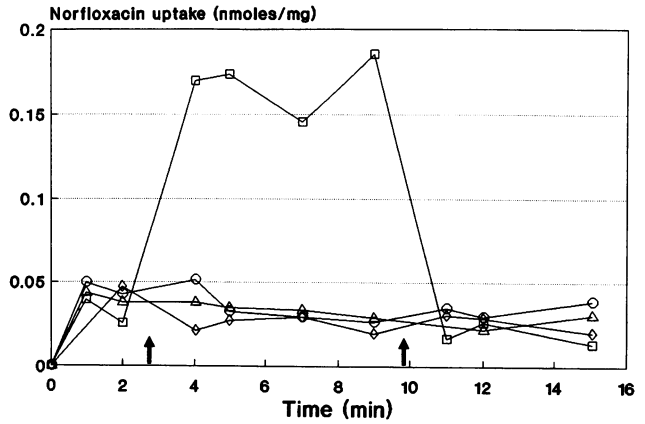

FIG. 5. Uptake of norfloxacin by everted inner membrane vesicles from *E. coli* DH10B containing plasmids pMT101 (○, □) and pUC19 (△, ◇). Norfloxacin was added at time zero. Lactate (20 mM) (□, △) or buffer (○, ◇) was added at the time indicated by the arrow on the left, and CCCP (100 μM) was added at the time indicated by the arrow on the right.

Role of magnesium in *norA*-mediated norfloxacin transport. The efflux of tetracycline by the Tet protein occurs as a complex of tetracycline with magnesium (37). Because quinolones also bind magnesium (35), we investigated the role of magnesium in *norA*-mediated norfloxacin uptake into everted vesicles. Vesicles were washed twice in KPO_4 buffer containing 10 mM EDTA. Results of uptake assays containing 0, 1, and 10 mM added magnesium were then compared. In seven paired comparisons in two experiments with EDTA-washed vesicles, uptake in the presence of 1 mM magnesium (mean ± standard deviation, 0.671 ± 0.121 nmol/mg of vesicle protein) was slightly, although significantly, greater than that in the absence of added magnesium (0.584 ± 0.13 nmol/mg) ($P = 0.02$; Student's t test), but uptake decreased in the presence of 10 mM magnesium (0.489 ± 0.132 nmol/mg) ($P = 0.014$) (Fig. 7). Thus, there is a slight (about 14%) enhancement of uptake in the presence of 1 mM magnesium, but most importantly, the majority of norfloxacin uptake does not depend on exogenously added magnesium.

Saturation kinetics of norfloxacin transport in everted vesicles. Energization of everted vesicles by lactate is accompanied by proton pumping into the vesicle, thereby lowering the internal vesicle pH. Because the microscopic dissociation constants of norfloxacin predict that, at equilibrium, drug will accumulate within vesicles in which the intravesicular pH is less than that of the medium (24), it is possible that drug uptake into vesicles results from diffusion and drug trapping rather than transport by a specific carrier. Thus, to determine whether the uptake of norfloxacin in vesicles prepared from *norA*-containing cells was carrier mediated and to investigate the characteristics of this putative carrier, we studied the saturation kinetics of norfloxacin uptake. In order to determine as closely as possible the initial rates of norfloxacin uptake, uptake was measured at 20°C, and initial samples were collected and diluted within 10 s of the addition of [^{3}H]norfloxacin. Initial rates of lactate-dependent uptake decreased

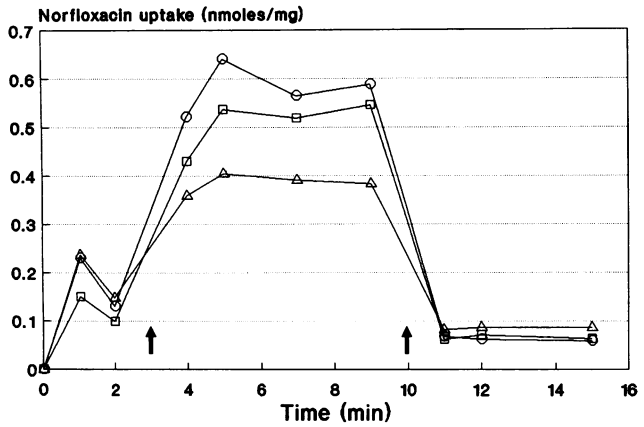

FIG. 7. Effect of magnesium supplementation on uptake of norfloxacin by everted vesicles from *E. coli* DH10B(pMT101). Vesicles were washed twice in 50 mM KPO_4 (pH 7.5)–10 mM EDTA and were resuspended in assay buffer containing 0 (□), 1 (○), and 10 (△) mM $MgCl_2$. Norfloxacin was added at time zero. Lactate (20 mM) was added at the time indicated by the arrow on the left, and CCCP (100 μM) was added at the time indicated by the arrow on the right.

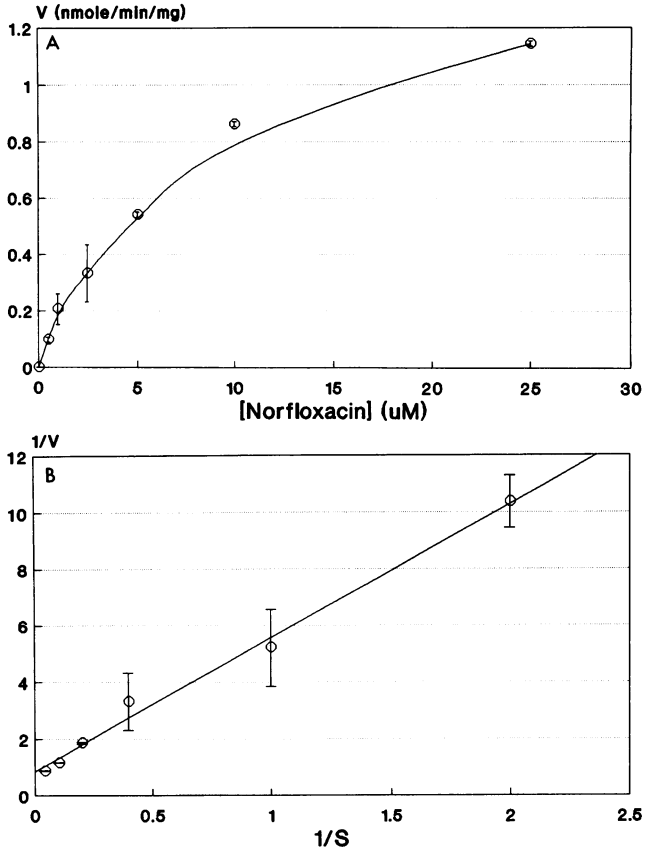

FIG. 8. Kinetics of initial uptake of norfloxacin by everted vesicles of *E. coli* DH10B(pMT101) as a function of the norfloxacin concentration. (A) Saturation curve. (B) Lineweaver-Burk plot. *V*, initial rate of uptake; *S*, norfloxacin concentration.

with increasing concentrations of norfloxacin over the range of 0.5 to 25 μM (Fig. 8A and B). For pMT101, the apparent K_m calculated from the mean of duplicate experiments was 6.0 μM from plots of $1/V$ versus $1/S$ (Fig. 8B) and 6.8 μM from plots of V versus V/S (data not shown). Similar values for K_m were found in experiments with pEN201 (data not shown). Values of V_{max} were 1.3 to 1.4 nmol/min/mg of vesicle protein for pMT101.

To assess whether the saturation of norfloxacin uptake resulted from an effect of the drug on the pH gradient across the vesicle membrane, we measured lactate-induced quenching of acridine fluorescence in vesicle preparations in the presence and absence of norfloxacin at high concentration (400 μM). No change in the observed fluorescence quenching was seen (data not shown), a finding consistent with results of previous studies with *E. coli* (4). Because artifactual saturation of uptake might occur if drug transport resulted in the development of an electrical gradient that limited the rate of uptake at high drug concentrations, we also measured the rate of norfloxacin uptake in the presence of valinomycin saturated with K^+, which dissipates the electrical gradient. Saturation of uptake was also seen in the presence of valinomycin (Fig. 9A and B). Thus, *norA* produces a saturable, carrier-mediated uptake of norfloxacin into everted vesicles.

Competition for norfloxacin uptake by other quinolones. Cloned *norA* and the *flqB* mutation cause resistance to the quinolones norfloxacin, ciprofloxacin, and ofloxacin to various extents. Of these quinolones, only norfloxacin was available in a radiolabeled form to a high specific activity. Thus, it was necessary to measure the transport of the other quinolones indirectly. To determine whether differences in NorA-mediated transport could account for these differences in quinolone activity when expression of *norA* is amplified, we examined the ability of ciprofloxacin and ofloxacin to compete with norfloxacin for transport into everted vesicles prepared from *norA*-containing cells.

Ciprofloxacin and ofloxacin both inhibited norfloxacin transport in an apparently competitive manner (Fig. 10A and B), but these two drugs differed in the magnitude of competitive inhibition. Ciprofloxacin was the more effective competitor (apparent K_i = 26.4 ± 4.4 μM [mean ± standard error of the mean]) (Fig. 10A), and ofloxacin was less effective (apparent K_i = 50.5 ± 9.6 μM) (Fig. 10B). Thus, the order in which these compounds competed with norfloxacin was the same as the order of the effect of *norA* on resistance in the cells used to prepare the vesicles (increase in MICs: ciprofloxacin, greater than eightfold; ofloxacin, fourfold [Table 3]). These correlations suggest that, for these three quinolones, a determinant of *norA*-mediated resistance is the ability of NorA to transport these compounds.

Effects of reserpine and verapamil on norfloxacin uptake into everted vesicles and norfloxacin resistance in *flqB* mutants. Reserpine and verapamil have been shown to inhibit the multidrug resistance mediated by the chromosomal *bmr* gene and cloned *norA* in *Bacillus subtilis* (20, 21). To determine the effects of these inhibitors in everted vesicles, norfloxacin uptake was measured in the presence of reserpine (Fig. 11) and verapamil (data not shown). At concentrations less than those that inhibit Bmr-mediated drug resistance in *B. subtilis* (21), reserpine (4.1 and 8.2 μM) and verapamil (11 μM) completely inhibited norfloxacin uptake into vesicles. Concentrations of reserpine of ≤0.4 μM produced no detectable inhibition.

To determine further the effect of reserpine on resistance in *S. aureus*, the MIC of norfloxacin for ISP794 (wild type) and MT23142 *flqB* was determined in Penassay broth with and without reserpine (Table 4). Reserpine (10 μg/ml, 16.4 μM) decreased the MIC of norfloxacin for MT23142, but it had a

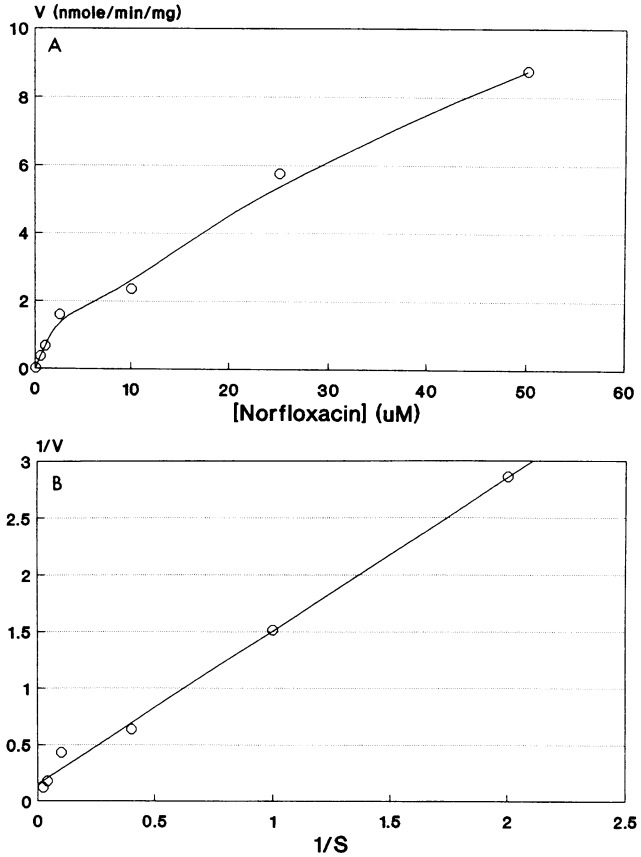

FIG. 9. Saturation kinetics of norfloxacin uptake by everted vesicles of *E. coli* DH10B(pMT101) in the presence of valinomycin (0.36 μM). (A) Saturation curve. (B) Lineweaver-Burk plot. *V*, initial rate of uptake; *S*, norfloxacin concentration.

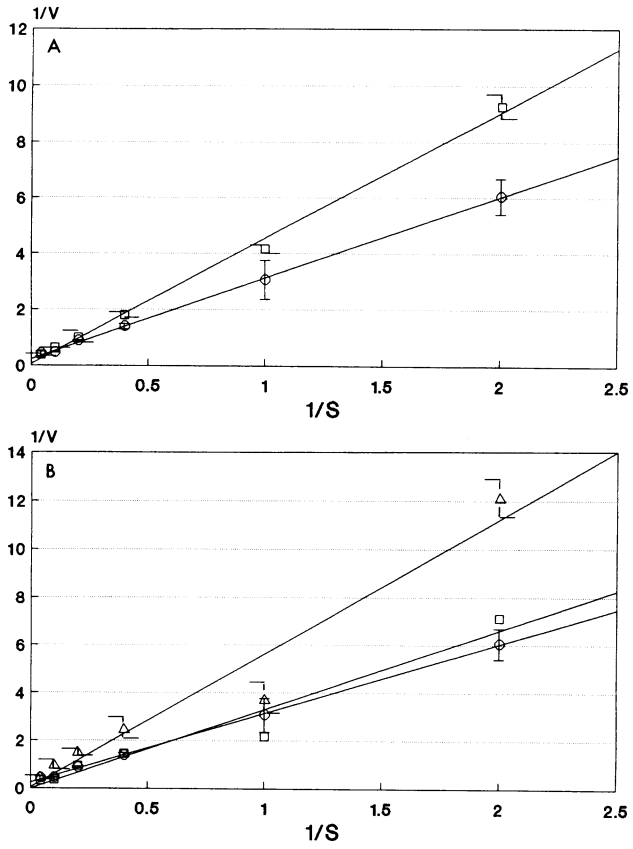

FIG. 10. Inhibition of norfloxacin uptake into everted vesicles of *E. coli* DH10B9(pMT101) in the presence of ciprofloxacin (A), at 0 μM (○) and (□) 15 μM (□) and ofloxacin (B) at 0 μM (○), 15 μM (□), and 45 μM (△). *V*, initial rate of uptake; *S*, norfloxacin concentration.

minimal effect on the MIC of norfloxacin for ISP794. In addition, reserpine had little effect on the norfloxacin MIC for resistant *flqA* mutants, indicating that the effect of reserpine is specific for *flqB* and is not an effect on quinolone resistance loci in general.

DISCUSSION

We identified *flqB*, a new quinolone resistance locus on the *S. aureus* chromosome, which is linked to *Ω1108* and *fus* on the *Sma*I D fragment, the same fragment that contains the *norA* gene (36). Cloning and nucleotide sequencing of the *norA* gene from *flqB* and *flqB*$^+$ strains identified a single nucleotide change 89 bp upstream from the putative ATG start codon. This mutation is located in a region between the *norA* gene and the putative −10 and −35 sequences that may function as a *norA* gene promoter (39). These findings indicate that resistance is not due to changes in the NorA protein. Resistance was, however, associated with a single nucleotide change in the putative promoter region, which may be responsible for the increased steady-state levels of *norA* mRNA observed in the *flqB* mutant. The present evidence suggests that resistance may occur from increased levels of expression of *norA* either by the increased levels of transcription or by the increased stabilities of *norA* transcripts. Our finding that strains containing *norA* genes cloned onto high-copy-number plasmids from wild-type and *flqB* strains express equivalent levels of resistance is also consistent with the concept that expression of wild-type *norA* is sufficient to cause resistance. Our findings also confirm a recent report of increased levels of *norA* transcripts in a resistant clinical strain for which no genetic data were available (13). In drug-resistant *bmr* mutants of *B. subtilis*, increased levels of expression are associated with duplications of the *bmr* gene on the chromosome (21). To check for *norA* gene duplication in *flqB* mutants, we probed *Eco*RI and *Hin*dIII digests of genomic DNAs from ISP794, MT23142, and MT1222 with a 4.3-kbp fragment containing *norA* and found no substantial differences in band intensity and no new bands (data not shown), suggesting that *flqB* mutants do not contain *norA* gene duplications.

Our *norA* sequences differed at several positions from those of clinical strains published previously (26, 39). The *norA* sequence of a resistant clinical isolate determined by Yoshida et al. (39) was identical to our sequence of wild-type ISP794 except for a substitution of a G for an A at position 872 that encoded a change from aspartic acid (Asp) to glycine (Gly) at codon 291 (Gly-291). Gly-291, however, was also reported to be present in the partial sequence from another quinolone-susceptible strain reported by Ohshita et al. (26), suggesting that a change to Gly-291 may not be responsible for *norA*-mediated resistance. Ohshita et al. (26) reported the *norA* sequence from positions +686 (by our numbering) to beyond the coding sequence at position +1284. Within this region there were five additional differences between the sequence of our wild-type ISP794 and that of their norfloxacin-susceptible

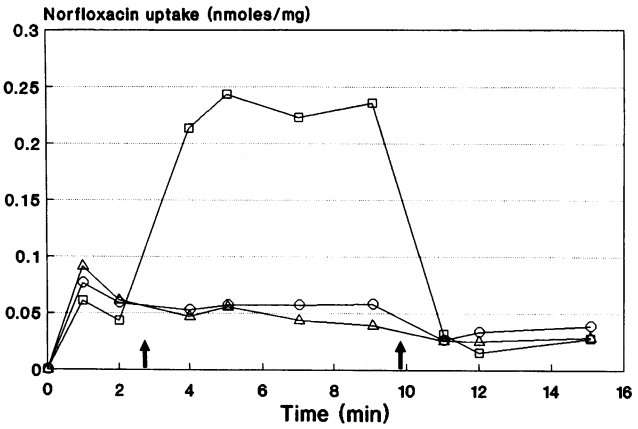

FIG. 11. Effect of reserpine at 4.1 μM (○) and 8.2 μM (△) in comparison with that of the diluent control (□) on the uptake of norfloxacin by everted vesicles of *E. coli* DH10B(pMT101). Inhibitors were added 5 min prior to the addition of norfloxacin at time zero. Lactate (20 mM) was added at the time indicated by the arrow on the left, and CCCP (100 μM) was added at the time indicated by the arrow on the right.

strain, two of which were within the *norA*-coding region. At position 787, a G replaced an A, resulting in the replacement of a valine for a methionine at codon 263, and at position 1085, an A replaced a C, resulting in the replacement of an Asp for an Ala at codon 362. Between nucleotide positions +686 and +1284, the resistant and susceptible strains reported by Ohshita et al. (26) differed only in a deduced change from Asp (in the susceptible strain) to Ala (in the resistant strain) at codon 362. Our finding of Ala-362 in the deduced amino acid sequence of NorA from the wild-type susceptible strain ISP794, however, casts doubt on previous claims that Ala-362 is responsible for quinolone resistance (26). Another *norA* allele from a susceptible clinical strain with an overall greater divergence of sequence has also been shown to encode Ala-362 (13). Thus, changes in NorA structure are not necessary for resistance, and increased levels of expression of wild-type *norA* appear to be sufficient.

Cloned *norA* causes an energy-dependent, reduced level of drug accumulation in intact *E. coli* (12, 39) and *S. aureus* (39) cells. Studies of quinolone uptake into everted vesicles have been used to distinguish active efflux from active reduction of influx in whole cells (13). Because, however, the norfloxacin that enters everted vesicles by diffusion may be trapped when the pH within the vesicle falls following energization with lactate (24), limited earlier experiments demonstrating norfloxacin uptake upon energization of everted vesicles did not answer the question of whether transport is diffusional or carrier mediated. We have demonstrated for the first time that cloned *norA* mediates drug uptake into everted vesicles and that this drug uptake is saturable, with an apparent high affinity for norfloxacin (K_m = 6 μM). Although it is possible that *norA* at a high copy number induces changes in the intrinsic *E. coli* membrane proteins that themselves mediate uptake, a simpler explanation is that NorA itself mediates uptake and functions as an efflux transporter. This presumption is also supported by the observation that *norA*-mediated norfloxacin uptake into everted vesicles has an apparent K_m value more than 10-fold less than that reported previously for *E. coli* (4). It is of interest that the K_m of 6 μM for *norA*-mediated norfloxacin transport is between the MIC of norfloxacin for wild-type *S. aureus*

TABLE 4. Effect of reserpine on quinolone resistance in *S. aureus*

Strain	Genotype	Norfloxacin MIC (μg/ml)	
		Without reserpine	With reserpine
MT1222	*flqB flqA flqC*	256	64
MT23142	*flqB Ω1108*	8	2
MT52222	*flqA541*	8	8
MT5224c9	*flqA543*	8	8
ISP794	Wild type	1–2	1

ISP794 (0.5 μg/ml, 1.6 μM) and the *flqB* mutant MT23142 (4 μg/ml, 12.5 μM), suggesting that NorA functions in vitro within a range of drug concentrations that is relevant for affecting drug action on the intact cell in vivo.

Other quinolones appear to cause competitive inhibition of *norA*-mediated transport of norfloxacin into vesicles. The apparent affinities of ciprofloxacin and ofloxacin for NorA, as estimated from their apparent K_i values, correlate with the extent to which *norA* effects resistance to these compounds. Thus, efflux transport of these quinolones appears to be a determinant of *norA*-mediated resistance.

Inhibition of *norA*-mediated norfloxacin transport by CCCP and nigericin but not valinomycin indicates that norfloxacin efflux is coupled to the proton gradient across the cell membrane. Thus, *norA* likely produces norfloxacin:proton antiport, and NorA itself is likely the antiporter. The stoichiometry of the coupling of norfloxacin and proton transport remains to be studied.

The plasmid-encoded TetB protein, which also contains 12 deduced membrane-spanning domains, mediates Mg-tetracycline:proton antiport but has limited amino acid sequence similarity with NorA. Interestingly, the region of Tet presumed to represent a cytoplasmic loop between transmembrane segments 2 and 3, which is important for tetracycline transport, is conserved in NorA and other related transporters (29). In contrast, however, many of the charged amino acids located within the stretches of hydrophobic amino acids thought to represent transmembrane segments differ. Tet and NorA also differ in their profiles of antibacterial resistance, because cloned *norA* does not encode tetracycline resistance (22). Norfloxacin transport by NorA and the transport of tetracycline by the Tet protein differ in their dependence on magnesium. Under conditions (EDTA washes) that were at least as rigorous in removing magnesium as those used to remove magnesium for testing vesicles containing Tet protein (18), norfloxacin transport by *norA* vesicles was little affected by the absence of added magnesium.

Inhibitors of drug efflux systems have been shown to increase cell susceptibility to the effluxed drug. In particular, in mammalian cells, calcium channel blockers and other lipophilic cations inhibit the efflux of antitumor agents by the P glycoprotein, the *mdr* gene product, and block the resistance of cells with increased levels of expression of *mdr* (25). Although *mdr* and *norA* do not share homology, *norA* is similar to the *bmr* gene of *B. subtilis*, which encodes multidrug resistance that includes resistance to quinolones, and is inhibited by reserpine and verapamil (21, 22). We have shown here that the quinolone resistance of an *S. aureus flqB* mutant is specifically reversed by reserpine and that *norA*-mediated transport of norfloxacin in everted vesicles is blocked by reserpine and verapamil. Use of these and related inhibitors may provide important tools for defining the structure and function of NorA and determining its normal role in *S. aureus* as well as

for developing the means for circumventing *flqB*-type resistance in clinical settings.

Taken together, our results and those of others strongly suggest that *norA* encodes a fluoroquinolone efflux transporter and that its enhanced level of expression causes resistance, presumably by the active transport of norfloxacin and some other quinolones from the cell. The normal function of NorA is not known. Its role in quinolone efflux is likely incidental, because fluoroquinolones are synthetic agents, but it might function as a more general transporter of environmental toxins, suggesting that its expression might respond to environmental signals. In *E. coli*, drug resistance associated with reduced drug permeation, including permeation of fluoroquinolones, appears to involve a complex regulatory network that responds to a variety of environmental signals and that is mediated by the *mar* and *sox* regulons (2, 3). Although reduced permeation in gram-negative bacteria is effected in part by reduced numbers of porin diffusion channels, resistance associated with *marA* mutants also involves increased levels of expression of a tetracycline efflux transporter (7).

In gram-positive bacteria, the absence of an outer membrane may result in increased dependence on active drug efflux mechanisms of reduced drug or toxin permeation. On the basis of its deduced structure, NorA is a member of a growing family of evolutionarily related transport proteins containing 12 (NorA, TetB, Bmr, CmlA, Mtx) and 14 (QacA, TcmA) predicted transmembrane segments with a range of identified substrates including, in addition to fluoroquinolones, tetracyclines, chloramphenicol, quarternary ammonium compounds, rhodamine, puromycin, and ethidium bromide, among others (15, 23, 29). Among these transporters, NorA is structurally and functionally most similar to Bmr (20). For both transporters, drug resistance appears to be associated with increased levels of gene expression, by gene amplification in the case of *bmr* and likely by increased levels of transcription for *norA*. Whether these transporters are normally regulated by environmental signals is unknown. Further studies of the regulation of *norA* and *bmr* expression may be useful in defining their normal functions in the bacterial cell.

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